Δημιουργία Οπτικού Σήματος Σε Βιολογικά Δείγματα Μέσω Φθορισμού

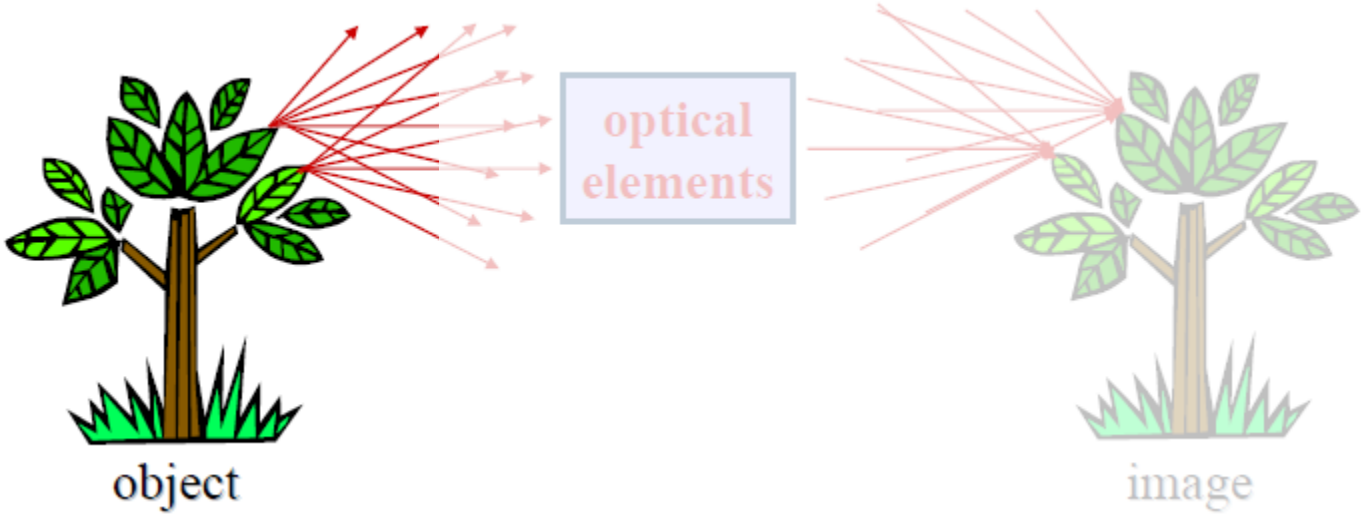
Δημήτριος Τζεράνης, Ph.D.

Εμβιομηχανική και Βιοϊατρική Τεχνολογία
Τμήμα Μηχανολόγων Μηχανικών | Ε.Μ.Π.

Χειμερινό Εξάμηνο 2015

The Three Components of Imaging

- Three major components



Emission generation
- Fluorescence

Optical system
- Microscope
- Endoscope

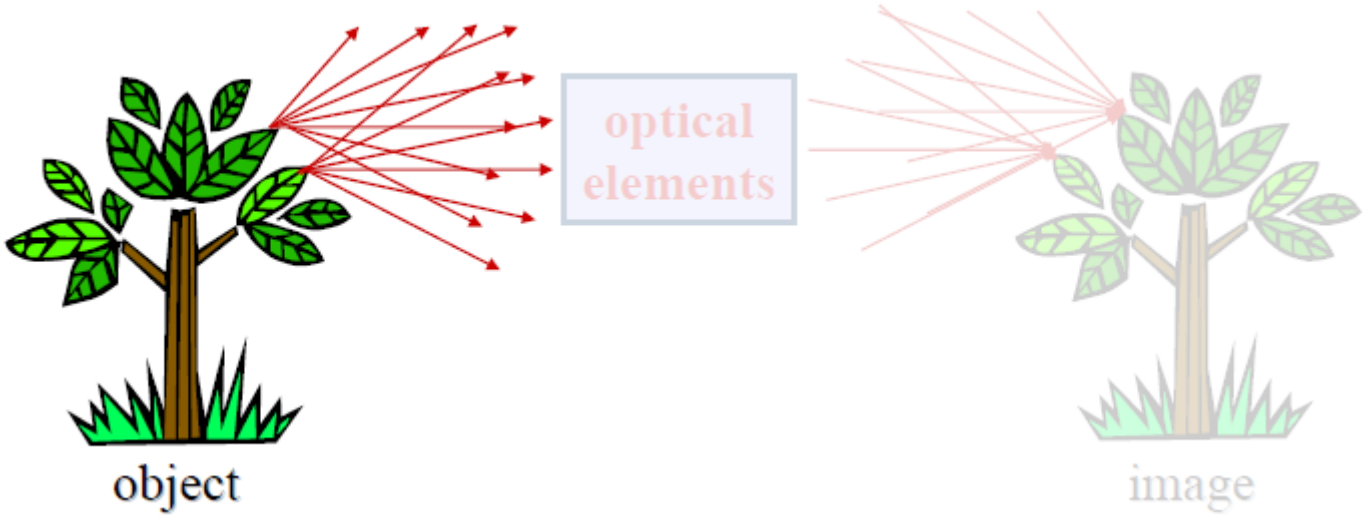
Emission detection
- Detector & sampling

Image by G. Barbastathis, MIT

Presentation Overview

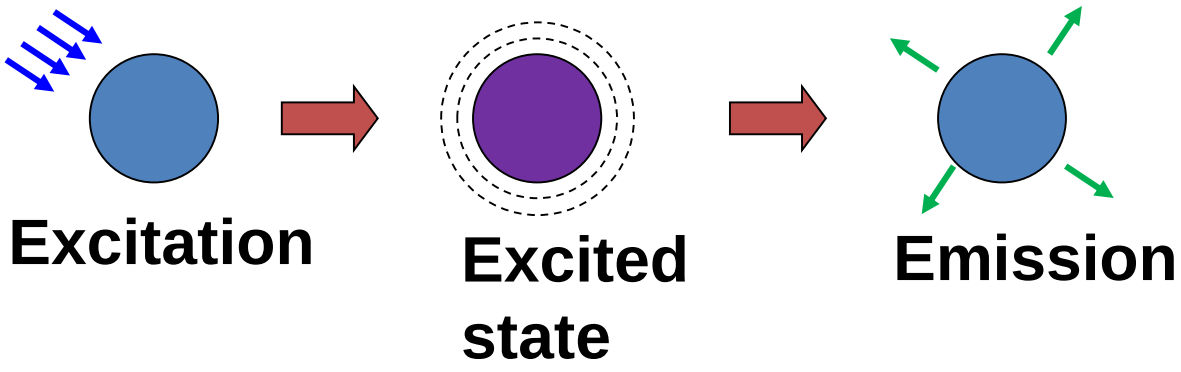
- Introduction to fluorescence
- Methods of fluorescent staining
- Förster sensors
- Key Applications of Fluorescence

Intro to Fluorescence



Fluorescence

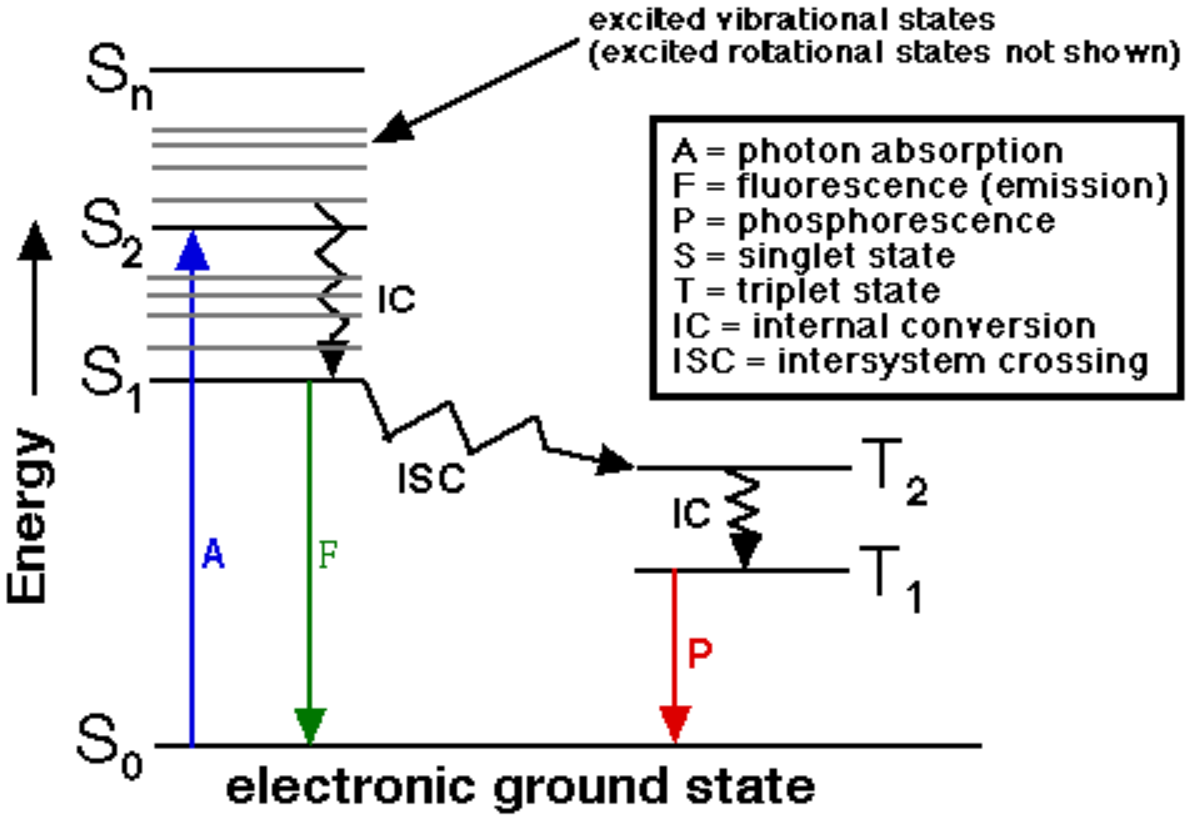
- An optical phenomenon, where a molecule (fluorophore) absorbs a photon, gets excited, and shortly later emits a photon of less energy and returns to ground state



- Key properties of each fluorophore
  - Absorption spectrum
  - Emission spectrum
  - Emission lifetime

Fluorescence

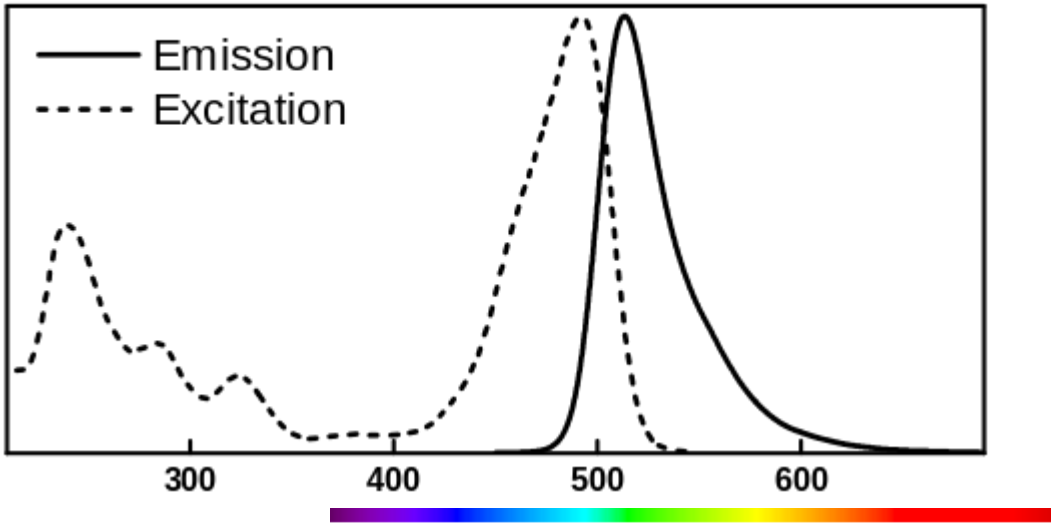
- Fluorescence absorption and emission is described by a Jablowski energy diagram



http://www.shsu.edu/~chm_tgc/chemilumdir/JABLON.GIF

Absorption and Emission Spectra

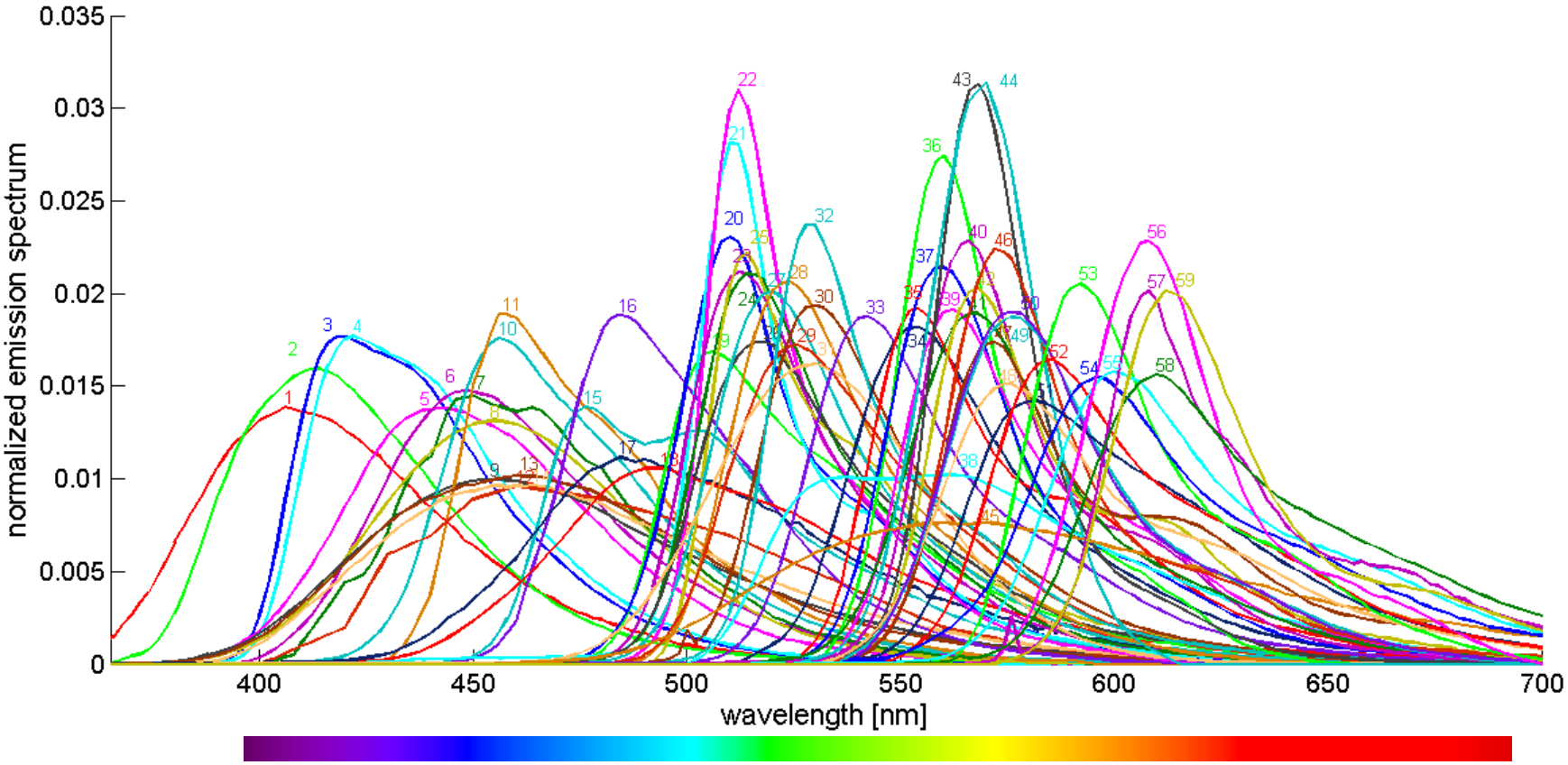
- Absorption spectrum $\alpha(\lambda)$: describes the ability of light of wavelength λ to excite the fluorophore
- Emission spectrum $\varepsilon(\lambda)$: describes the probability that a photon emitted by the fluorophore will have wavelength λ
  - $\varepsilon(\lambda)$ is always red-shifted compared to $\alpha(\lambda)$



www.Invitrogen.com

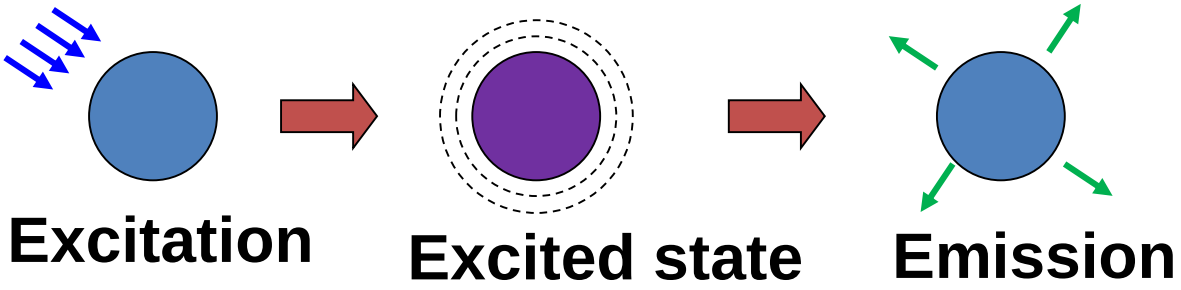
Absorption and Emission Spectra

- The spectra $\alpha(\lambda)$, $\varepsilon(\lambda)$ are properties of each fluorophore

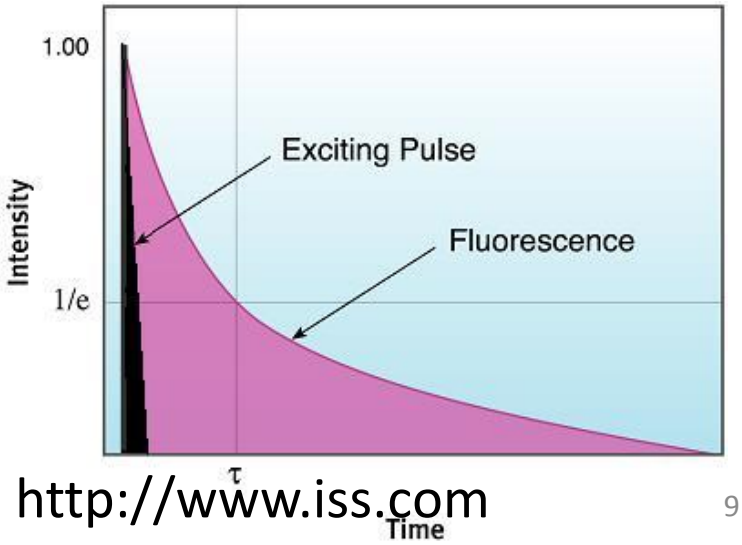


Fluorescence Lifetime

- There is a finite time delay between photon absorbance and photon emission

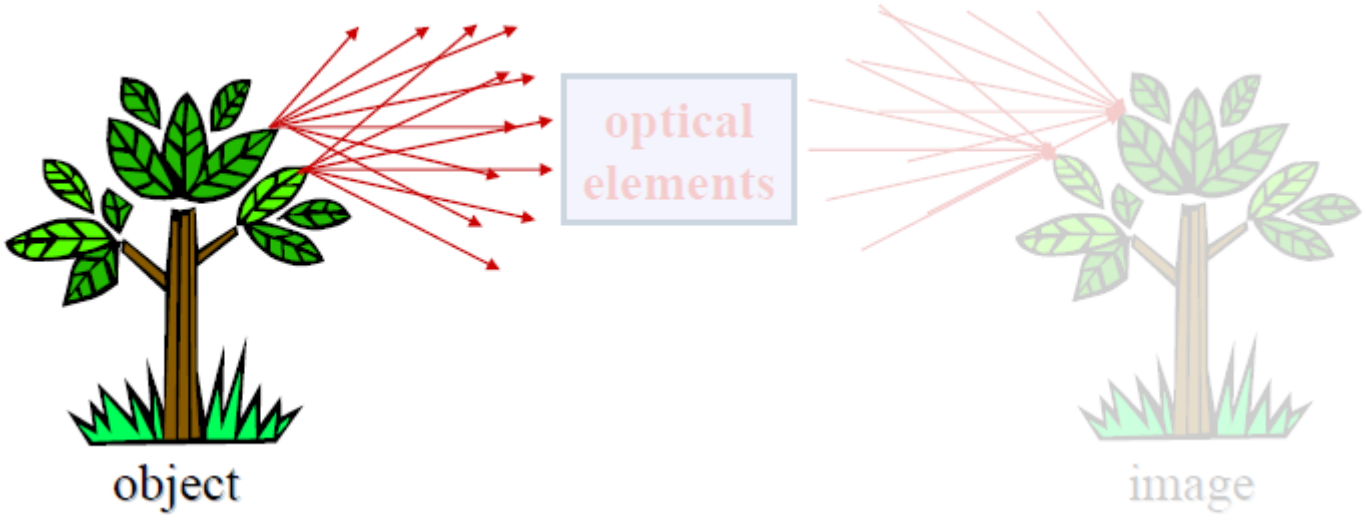


- This delay follows an exponential probability distribution with parameter τ: lifetime
  - Characteristic of a fluorophore
  - On the order of 1 nsec



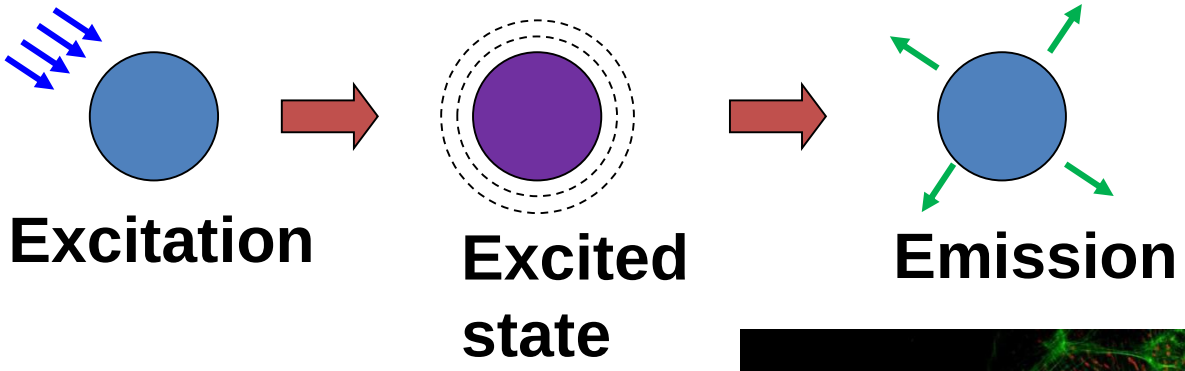
http://www.iss.com

Fluorescence Staining

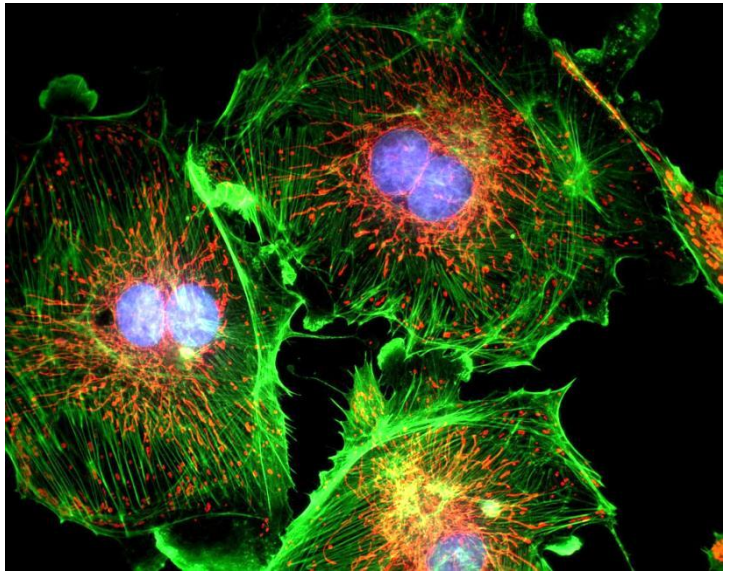


Overview: Fluorescence in Biological & Medical Imaging

- Fluorescent molecules (fluorophores) can be visualized using microscopes (see next presentation)



- Utilize fluorophores to label structures or proteins of interests *in vitro* or *in vivo*



www.dvcco.com

Types of Fluorophores Utilized in Biological & Medical Imaging

- Tissues/cells contain molecules that are fluorescent
  - Autofluorescence

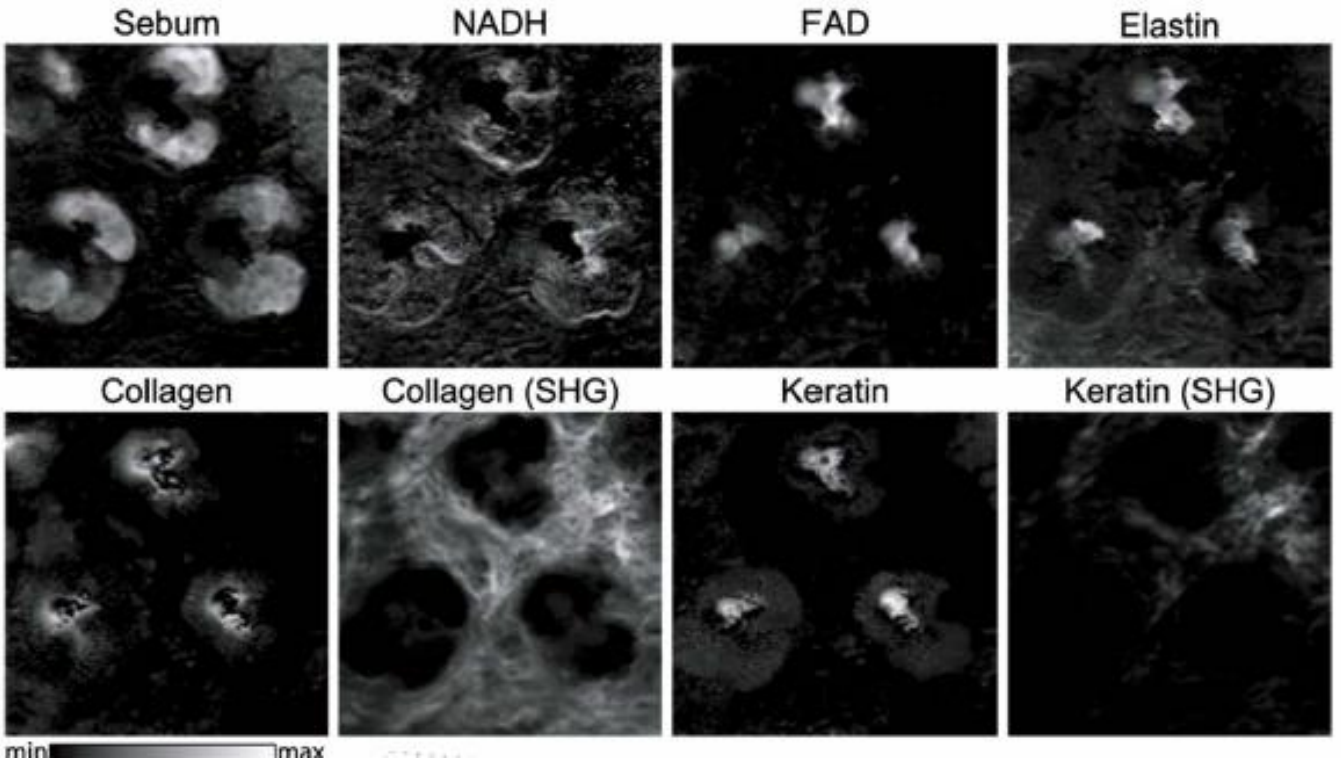
- Usually scientists add molecules that fluorescently stain/tag a molecule/structure of interest
  - Fluorescent Stains
  - Fluorescently-conjugated antibodies (immunofluorescence)
  - Fluorescent proteins
  - Fluorescent labeling of peptide tags

Intrinsic Fluorescence of Tissues & Cells (Auto-fluorescence)

- Cells/tissues contain several weakly-fluorescent molecules

Molecule
Tyrosine
Tryptophan
Serotonin
Melatonin
5-HIAA
5-HTOL
Retinol
Flavins
NADH
Pyridoxine
Folic acid
Cholecalciferol
Elastin
NFTs
Lipofuscin
Collagen
Microtubules
Skel. muscle

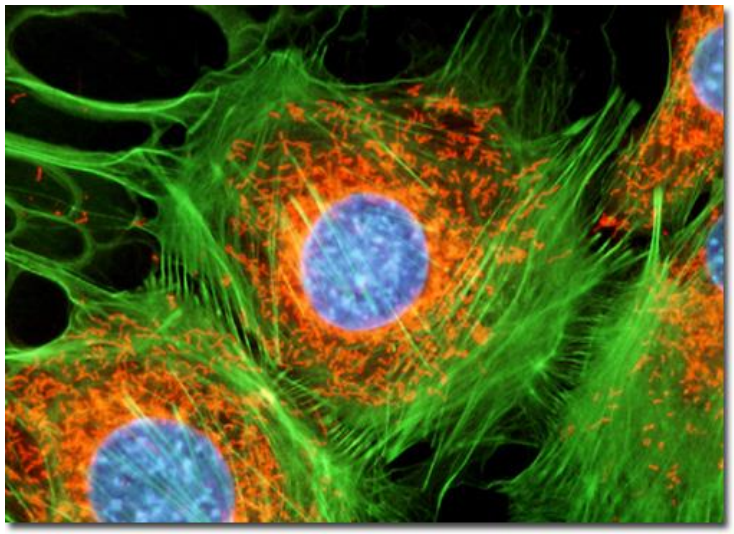
Key intrinsic fluorophores found in tissues (Zipfel et al. 2003)



Imaging skin *in vivo* using intrinsic fluorophore (Radosevich et al.2008)

Fluorescent Stains

- Chemical compounds that bind specifically to particular molecules found in tissues
  - Nucleic Acid Stains (e.g. DAPI)
  - Actin Stains (phalloidin)
  - Microtubules (paclitaxel)
  - Mitochondria (mitotracker)
  - Cytosol (cell trackers)
  - Cell membrane (WGA)
  - Live VS Dead cells

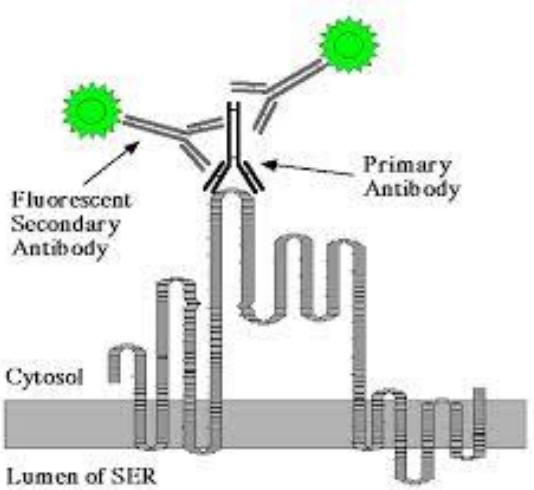


3T3 cells stained with DAPI (blue), phaloidin (green), and mitrotracker (red) (Nikon)

- Cells add the dye to sample → dye diffuses and binds target
- Applied extensively in cell imaging (*in vitro*) or *ex vivo* tissue imaging (thin sections)

Immunofluorescence (IF)

- Utilize a fluorescently-conjugated secondary antibody
  - Binds primary antibody that has bound target protein of interest



www.bio.davidson.edu

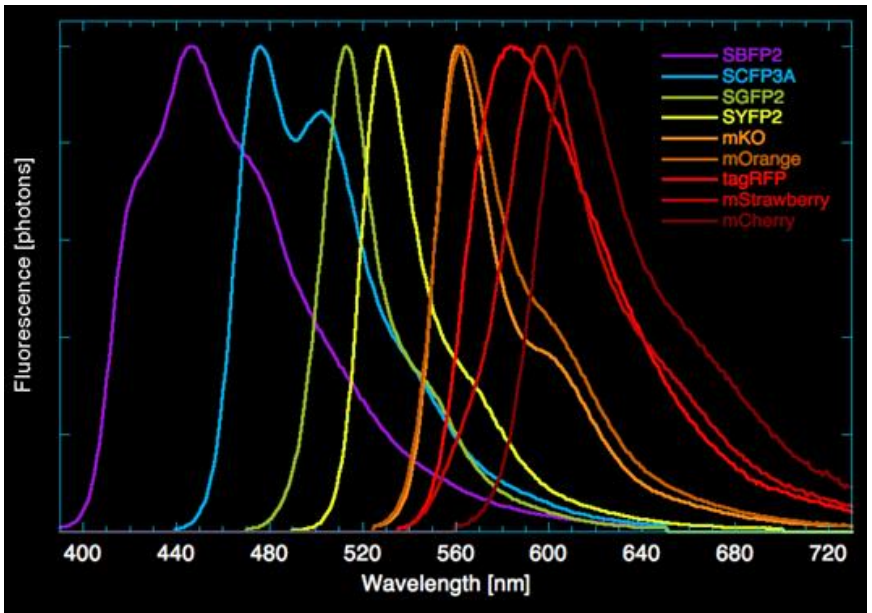
- Stain particular proteins in fixed cells *in vitro* or thin tissue sections *ex vivo*

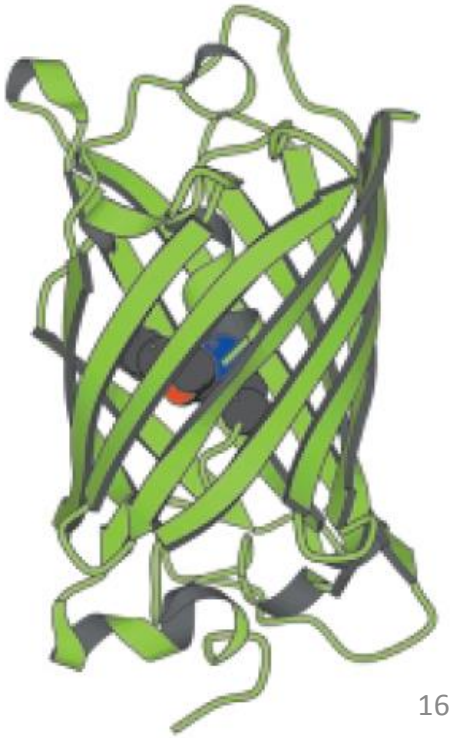
- Can use multiple (usually up to 3) sec. antibodies conjugated to different fluorophores

IF staining of vinculin (red), β-actin (green), and αSMA (blue) in contractile cells. Hinz et al. 2007

Fluorescent Proteins (FP)

- Intrinsically fluorescent proteins, aprox. 25 kDa size
- Green fluorescent protein (GFP) first discovered in Aequorea victoria in the 1960s
- Multiple mutants have developed with different properties
  - Emission spectrum (peak), monomeric, stability

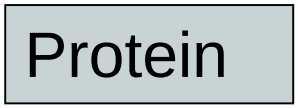




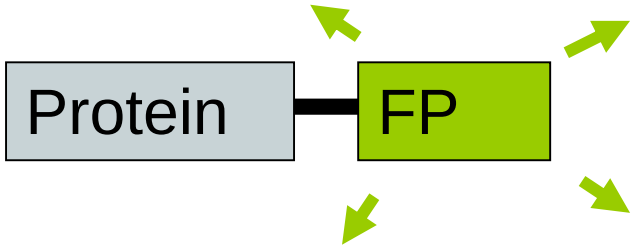
GFP domain
(Zhang, 2002)

Fluorescent Labeling via FPs

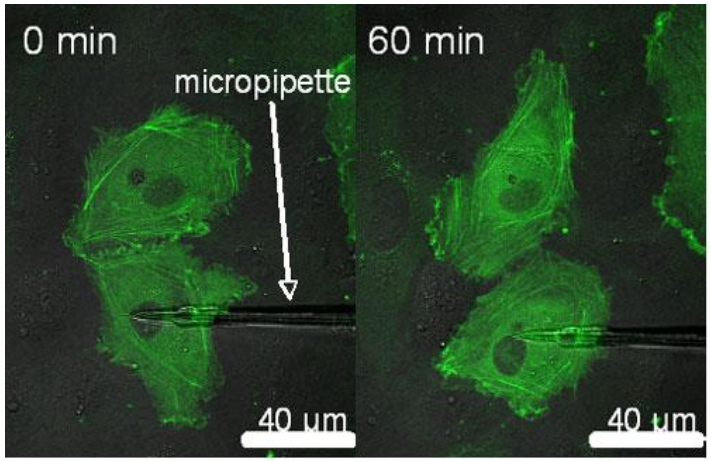
- Most proteins are not fluorescent



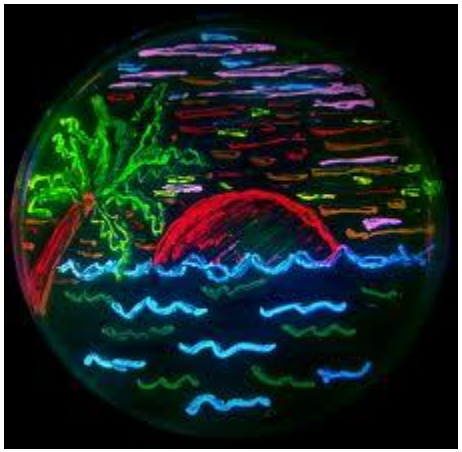
- Can make a protein fluorescent by fusing it with a FP



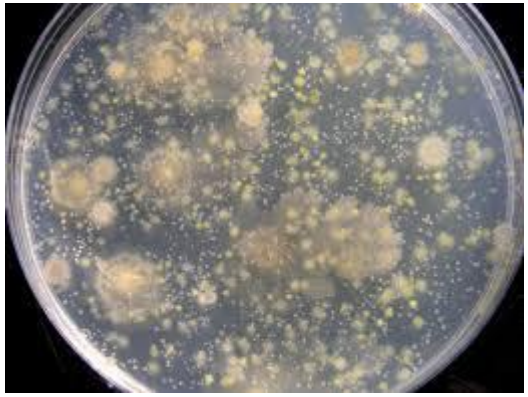
- Express in live cells *in vitro* or animals



http://www.nanobme.org/

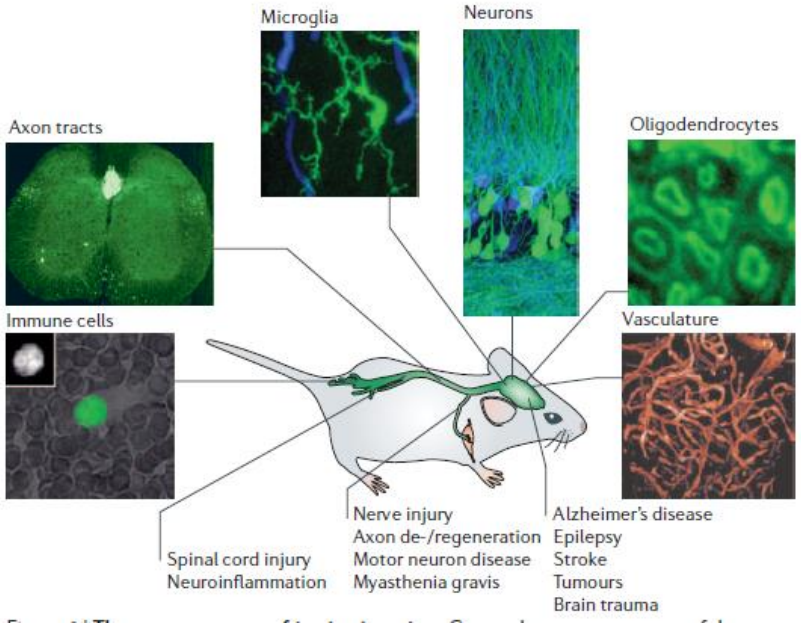


http://en.dogeno.us/

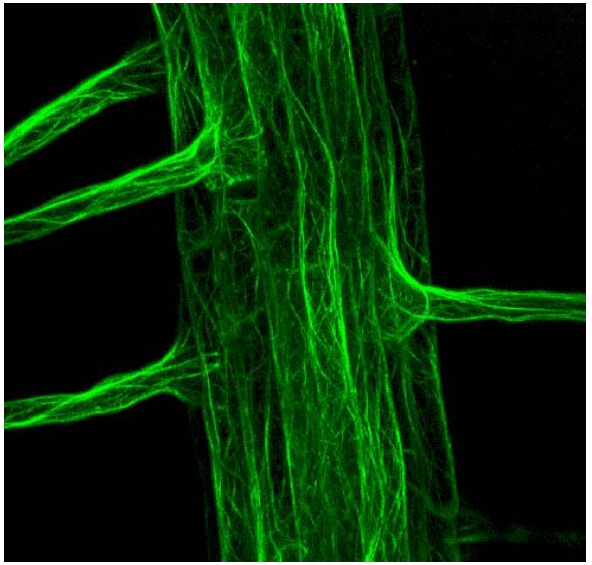


Examples of Application of FPs in Animals and Plants

- Generate transgenic animals/plants that express their native proteins fused to a FP
  - Need to incorporate the protein-FP fusion in the animal DNA
  - Enables *in vivo* imaging of particular markers!



Studying neurodegenerative diseases using GFP-mice and *in vivo imaging (*Misgeld at al. 2006)



Imaging fimbrin-GFP fusion in live Arabidopsis roots (http://www.noble.org/)

Nobel Prize in Chemistry 2008

The Nobel Prize in Chemistry 2008

Photo: U. Montan

Osamu Shimomura

Prize share: 1/3

Photo: U. Montan

Martin Chalfie

Prize share: 1/3

Photo: U. Montan

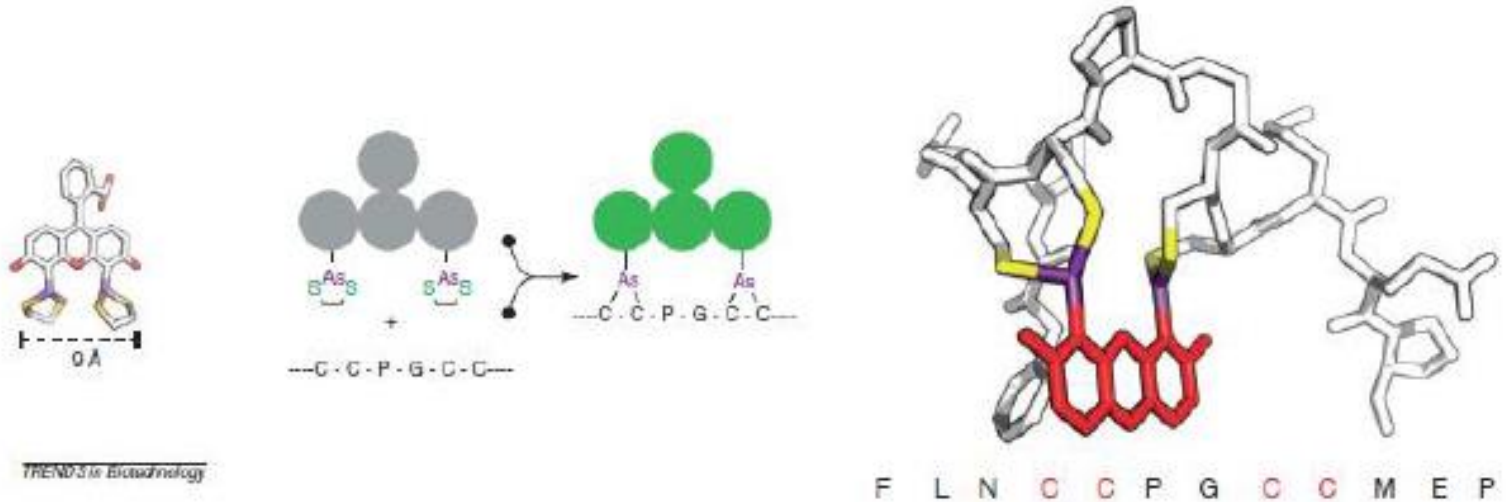
Roger Y. Tsien

Prize share: 1/3

The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien *"for the discovery and development of the green fluorescent protein, GFP".*

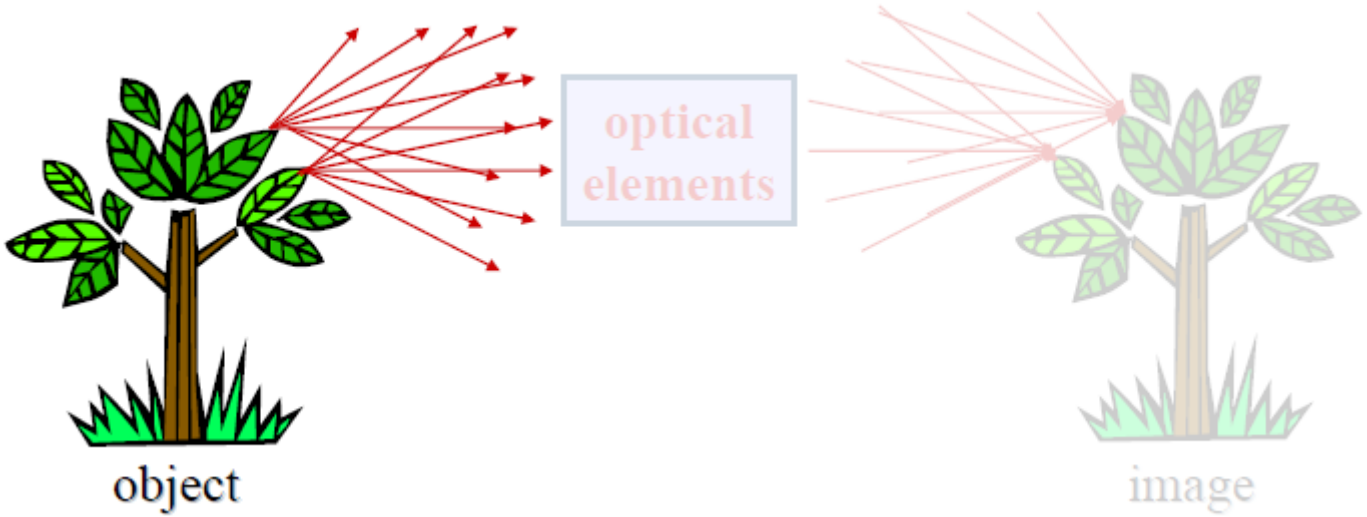
Fluorescent Labelling via Small Peptide Tags

- Fusing a FP with a protein may affect its function
- Alternative tag a protein with a small peptide, that becomes fluorescent upon binding to a small molecule
  - Examples: FlAsH – tetracysteine system



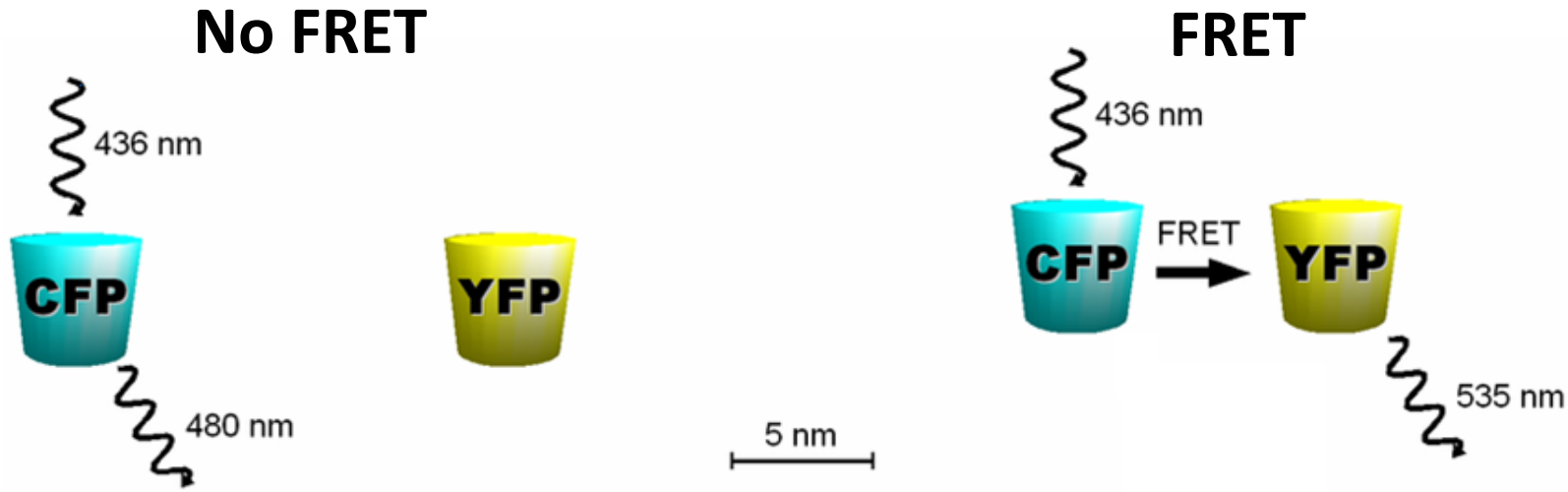
Fluorescent staining of proteins using the FlAsH dye (Madani et al. 2009; Crivat and Taraska 2012)

FRET Sensors



FRET Principle

- Förster resonance energy transfer (FRET) refers to the energy transfer from a “donor” to an “acceptor” fluorophore when they are located <7 nm away
  - Condition for FRET: donor ε(λ) of overlaps significantly with acceptor α(λ)

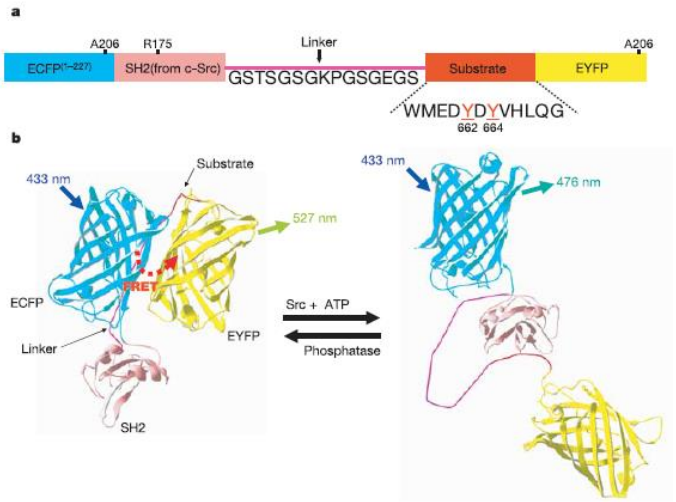


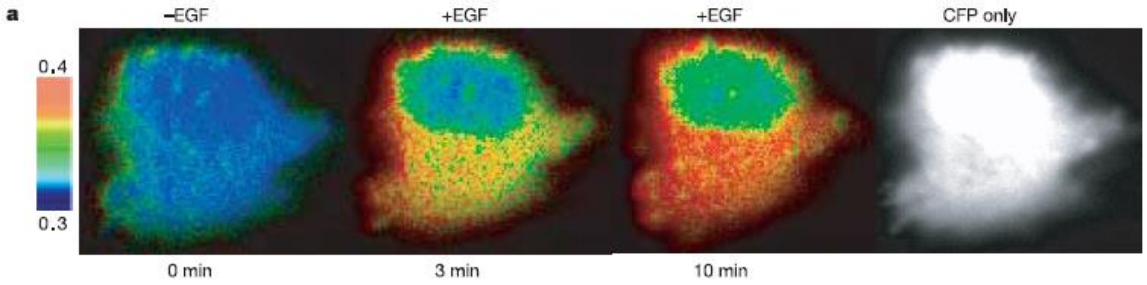
Normally, a blue laser excites CFP, but not YFP

When CFP, YFP are less than 7nm away → Blue laser excites YFP through CFP

FRET Sensors

- Sensors refer to artificially design fusion proteins where FRET takes place in ways that provide us information
  - FRET signal depends on protein conformation
  - Protein conformation depends on the entity we want to quantify
- FRET sensors can be expressed in live cells
  - Provide quantitative data *in vivo*

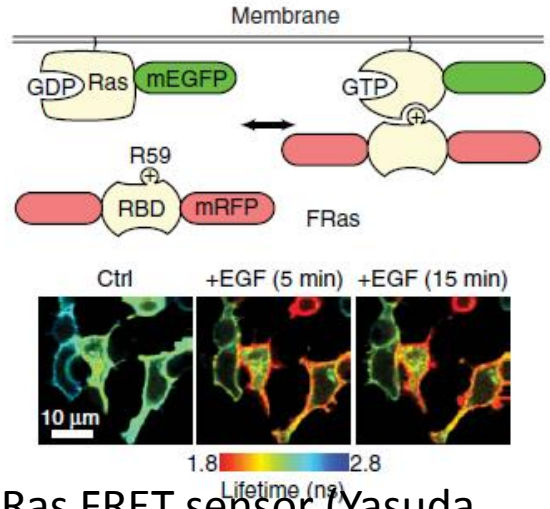




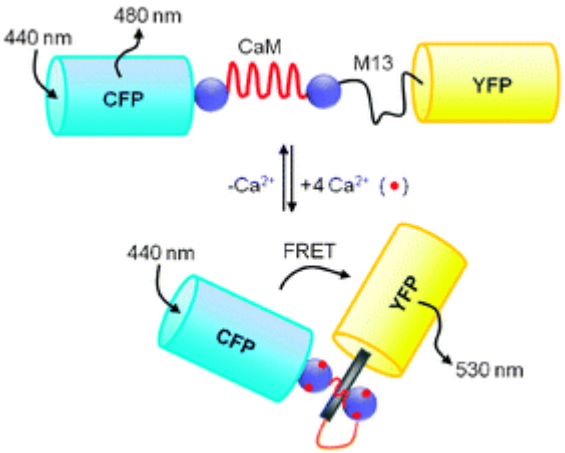
FRET sensor for quantifying the activity of Src small GTPase(Wang et al. 2005)

FRET Sensors

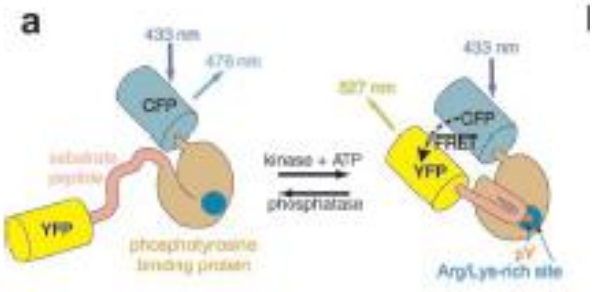
- Sensors designed so far can quantify:
  - Small GTPases
  - Kinase activity
  - Secondary messengers (Ca+2)
  - pH, cation concentration



Ras FRET sensor (Yasuda 2006)



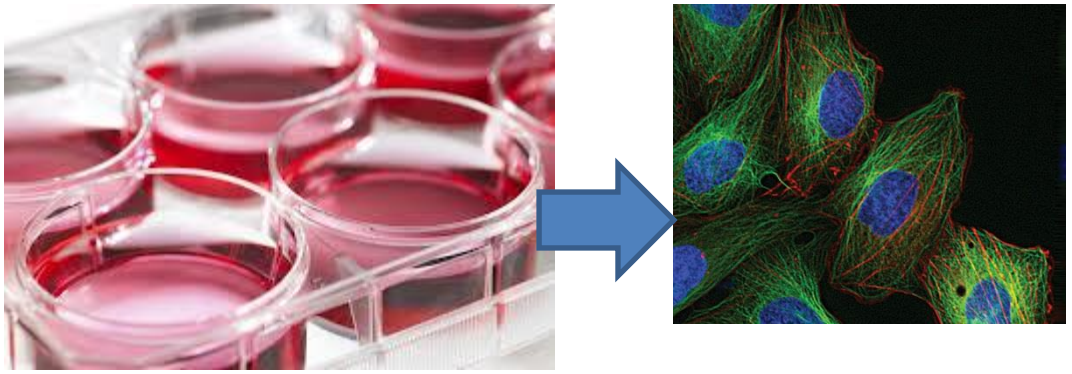
Ca++ FRET sensor (Ting2001)



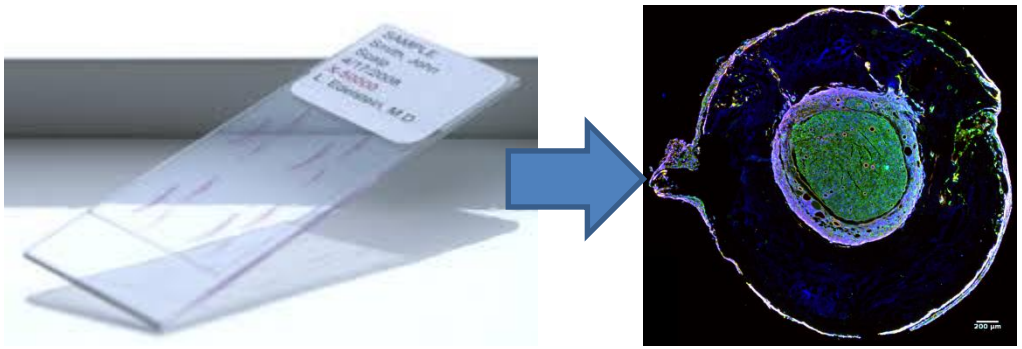
Kinase A FRET sensor (Ting2001)

Types of Samples in Biological Imaging

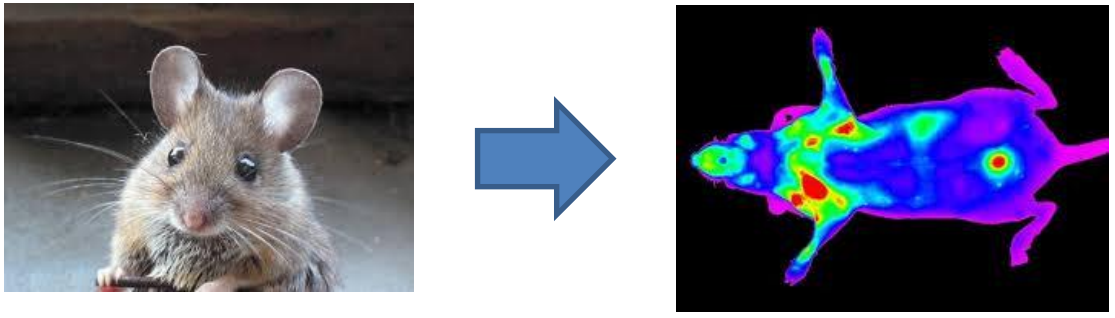
- Cells *in vitro*



- Tissue sections *ex vivo*



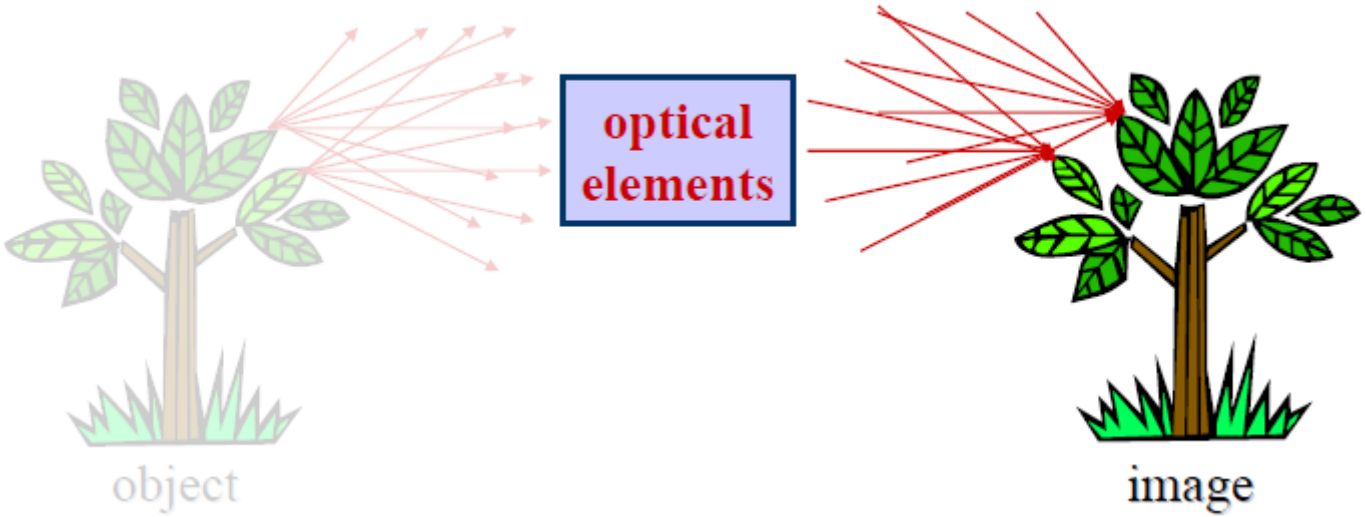
- Animal imaging *in vivo*



Whole Animal Imaging

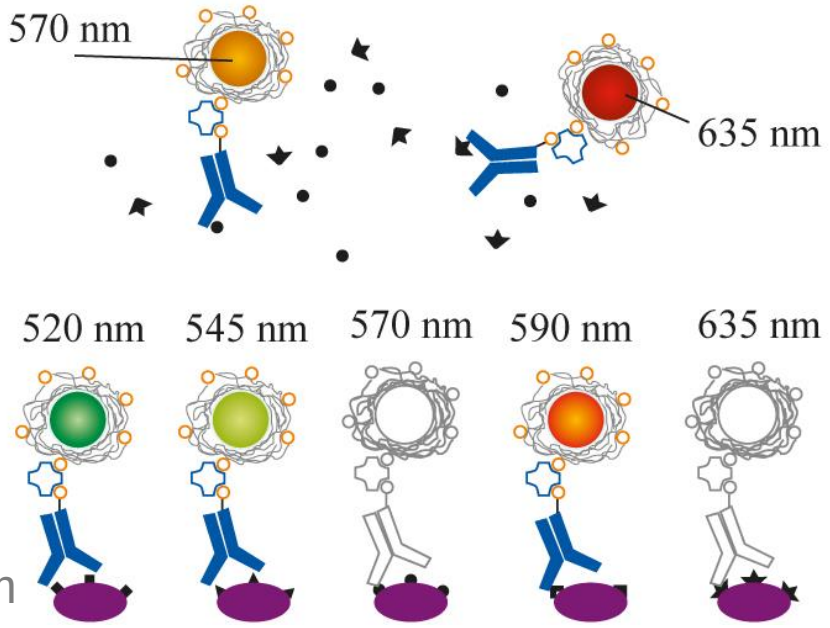
- Utilize fluorophores that emit in the IR
  - Inject/express in live animals
  - Excite using IR light
- Tomographic detection →computation →estimate the emission origin inside animal
  - Prof. V. Ntziachristos (Harvard U. , TUM)
- Utilized experimentally to study tumor progression

Major Applications of Fluorescence



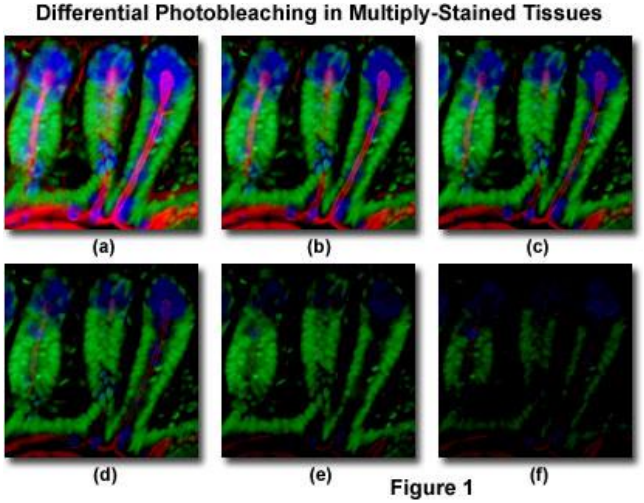
Fluorescence: Utilized in Detection

- Fluorescence provides a cheap and versatile way to detect the amount and spatial distribution of molecules that otherwise provide no optical signal…
  - Label molecule of interest with a fluorophore via the abovementioned methods (dyes, antibodies, proteins, tags)
  - Detect fluorophore via fluorescence → detect molecule of interest



Practical Aspects of Fluorescence Staining

- **Photobleaching** phenomenon : repetitive excitation of fluorophores leads to their eventual destruction
  - Prevent by: reducing illumination exposure, prevent O2 diffusion

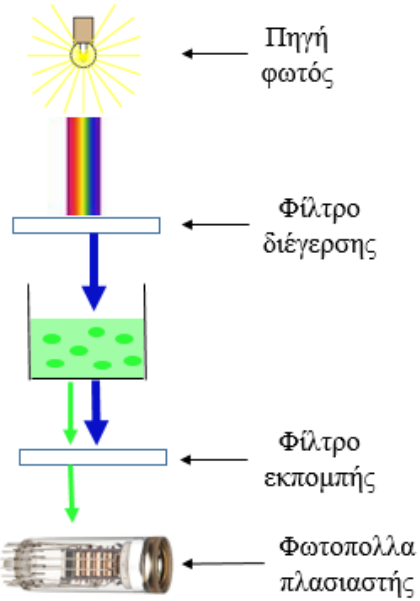


Photobleaching example (micro.magnet.fsu.edu)

- **Photodamage and phototoxicity**: once activated, flurophores may react with their environment→ cause damage to cells
  - Reduce illumination, use necessary staining

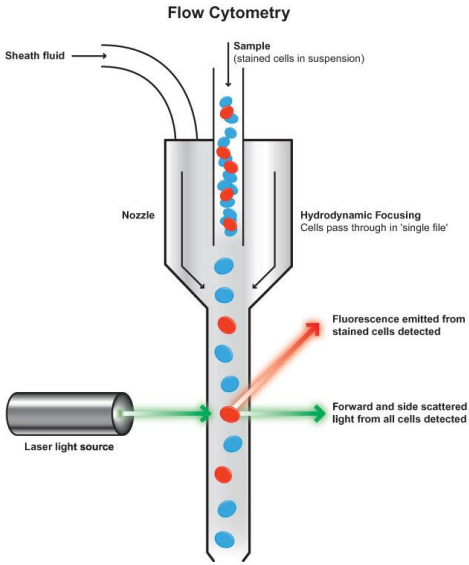
Key Instruments That Utilize Fluorescence in Biology & Medicine

- **Fluorescence platereader**
  - Cheap, widely utilized
  - Quantify solutions or cells



Biotek.de

- **Flow cytometer**
  - Quantify fluorophores in cells or beads



- **Fluorescence microscope**
  - see next…

Summary

- Fluorophores absorb photons of particular wavelength and within ~1nsec emit photons of less energy
- Tissues/cells contain several weakly fluorescent molecules (autofluorescence)
- Scientists label molecules of interest in cells/tissues using i) fluorescent stains, ii) fluorophore-conjugated antibodies, iii) fluorescent proteins
- Fluorescent proteins fused with proteins of interest in live cells and animals revolutionalized science
- The FRET phenomenon has been utilized to design “sensors” that can quantify the activity or concentration of various molecules *in vivo*
- Fluorescence labeling is utilized extensively to detect molecules that otherwise don’t emit optical signal